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RBMX is a novel hepatic transcriptional regulator of SREBP-1c gene response to high-fructose diet

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Abstract In rodents a high-fructose diet induces metabolic derangements similar to those in metabolic syndrome. Previously we suggested that in mouse liver an unidentified nuclear protein binding to the sterol regulatory element (SRE)-binding protein-1c (SREBP-1c) promoter region plays a key role for the response to high-fructose diet. Here, using MALDI-TOF MASS technique, we identified an X-chromosome-linked RNA binding motif protein (RBMX) as a new candidate molecule. In electrophoretic mobility shift assay, anti-RBMX antibody displaced the bands induced by fructose-feeding. Overexpression or suppression of RBMX on rat hepatoma cells regulated the SREBP-1c promoter activity. RBMX may control SREBP-1c expression in mouse liver in response to high-fructose diet.

Keywords: SREBP-1c; RBMX; hnRNPG; Fructose; Metabolic syndrome; RNA interference

1. Introduction

In rats a high-fructose diet causes metabolic derangements having characteristics resembling metabolic syndrome [1–3]. Rats fed high-fructose diet have been used as an animal model for metabolic syndrome presenting hypertriglyceridemia, high blood pressure, and impaired glucose tolerance [4,5]. These animals show elevated hepatic expression of sterol regulatory element (SRE)-binding protein-1c (SREBP-1c). SREBP-1c, which is one isoform of the SREBPs of the basic helix-loop-helix leucine zipper transcription factor family, plays a crucial role in the regulation of lipogenic enzymes in the liver. Because the elevation of SREBP-1c may play a key role in the pathogenesis of metabolic derangements in rats fed high-fructose diet [4,6], it is important to elucidate the molecular mechanisms by which the high-fructose diet induces SREBP-1c gene expression in the liver.

We previously reported a variation of susceptibility to these metabolic disorders after monitoring the effects of high-fructose diet in different mouse strains [6]. Ten strains of inbred mice were separated into CBA and DBA groups according to their response to high-fructose diet. The hepatic mRNA expression of SREBP-1c in CBA/JN mice was remarkably elevated by high-fructose diet but not in DBA/2N. We also observed a single nucleotide polymorphism (−468 G to A) at the promoter region of SREBP-1c, which caused impaired activation of SREBP-1c transcription in response to high-fructose diet. Interestingly, high-fructose diet induces the nuclear protein recognizing the region between −453 to −480 bp of SREBP-1c promoter, which includes the single nucleotide polymorphism at −468. These results suggest that this fructose-induced protein binding has a critical role in the transcriptional control of SREBP-1c gene associated with fructose feeding.

In this study, we attempted to identify this binding protein using MALDI-TOF MASS technique and identified it as RBMX (RNA-binding motif on the X chromosome, or heterogeneous nuclear ribonucleoprotein, hnRNPG). Here we show that RBMX, the newly identified nuclear protein, regulates the activity of SREBP-1c promoter induced by high-fructose feeding.

2. Materials and methods

2.1. Animals, cell culture, and preparation of nuclear protein extracts

Mice of the CBA/JN and DBA/2N strains (CLEA Japan Inc., Tokyo, Japan) were fed high-fructose diet or control diet (Oriental Yeast, Tokyo, Japan) for 8 weeks as previous described [6]. Fao hepatoma cells were maintained in DMEM with 10% FCS. Nuclear protein extracts from the livers of mice or from cultured cells were isolated as previously described [6].

2.2. Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as previously described [6] using radiolabeled double-strand oligonucleotides corresponding to the following pairs from the SREBP-1c promoter regions: DBA/2N mice, 5′-TATCTAAAGGCA*A*CTATTGG-3′ and 5′-GGAAGGCCAATAG*T*TG-3′; CBA/JN mice, 5′-TATCTAAAGGCAGCTATTGG-3′ and 5′-GGAAGGCCAATAGCTG-3′. For control study EMSAs were performed using radiolabeling OCT-1 consensus oligonucleotides (Promega). Anti-hnRNPG and anti-IgG antibodies were purchased from Santa Cruz. Competition assay using non-labeled oligonucleotides was also carried out.

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Abbreviations: EMSA, electrophoretic mobility shift assay; hnRNPG, heterogeneous nuclear ribonucleoprotein G; RBMX, RNA-binding motif protein on the X chromosome; RBMY, RNA-binding motif protein on the Y chromosome; RNAi, RNA interference; siRNA, small interference RNA; SREBP, sterol regulatory element (SRE)-binding protein

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2.3. DNA affinity purification of RBMX

DNA affinity purification of the proteins which bind to the SREBP-1c promoter regions was performed using double-strand oligonucleotides corresponding to the following pairs: Oligo-SRA 5′-AATTCCTAAAGGCAGCTATTGGCCTG-3′ and Oligo-SRB 5′-AATTCAGGCCAATAGCTGCCTTTAGG-3′. Annealed probes (1.5 nmol) were ligated with *Eco*RI-digested DNA fragments covalently bound to a resin (100 μl bed volume, Easy Anchor *Eco*RI-N from Nippon Gene). The resin preparation was incubated with 100 μg of nuclear protein extract from the livers of mice in the presence of 2 μg of poly(dI–dC) in buffer 1 (10 mM HEPES, pH 7.9, 60 mM KCl, 1 mM EDTA, 7% [w/v] glycerol) at 25 °C for 30 min. Then the resins were collected by centrifugation, washed five times with buffer 2 (15 mM HEPES, pH 7.9, 25 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% [w/v] glycerol) containing 0.1 M KCl, and eluted with buffer 2 containing 1.5 M KCl. Eluted fractions were centrifuged, and the supernatant was collected. The supernatant was purified using SDS/PAGE Clean-Up Kit (Amersham Biosciences) and then analyzed by SDS/PAGE. Proteins were visualized by silver staining. Two bands (42 and 43 kDa) were excised from the gel, digested with trypsin, and analyzed by MALDI-TOF MASS spectrometer (AXIMA-CFR). The resulting spectra were used to search for matching proteins in the NCBI database using the Mascot search program (Matrix Science).

2.4. Construction of RBMX expression plasmids

The 1.2-kb sequence of RBMX was amplified using cDNAs derived from mouse liver by PCR with the sense primer Rbmx-*Eco*RI 5′-GCGGAATTCAGGCAAGATGGTTGAAGCAG-3′ and the antisense primer Rbmx-*Xho*I 5′-GCGCTCGAGGCATGGAGACCTTGATCCAA-3′. The resultant amplicons were agarose gel-purified and inserted into pCRII-TOPO vector (Invitrogen) to form the RBMX-expressing plasmid, pcDNA-RBMX.

2.5. Cell transfection and luciferase assays

Transfections were performed using Superfect Transfection Reagent (Invitrogen) according to manufacturer's instructions. Transfections were carried out with 0.2 μg/well of pRSV-β-gal expression plasmid, 0.25 μg/well of the reporter plasmid, and pGL3-CBA/JN or pGL3-DBA/2N [6]. In the RBMX expression experiments, 0.75 μg/well of control pcDNA plasmid or pcDNA-RBMX expression plasmid was also transfected. The results were quantified with a luminometer and normalized to the β-galactosidase activity measured in the cell extract.

2.6. RNA interference

Small interference RNA (siRNA) oligonucleotides were designed by iGENE Therapeutics, Inc. (http://igene-therapeutics.co.jp/). Annealed RNA oligonucleotides encoding both the sense and antisense target sequences were from Hokkaido System Science. The siRNA sequence targeting rat RBMX (GenBank accession number NM_001025663) was 5′-AGAUUCGUAUGAGAGUUAUGGUAAC-3′ (corresponding to 846–871 nt of ORF), and that of mouse RBMX (GenBank accession number NM_011252) was 5′-CGAGAAACGAAUAAGUCAAGAGGAU-3′ (corresponding to 127–152 nt of ORF). Control (non-silencing) siRNA (QIAGEN) corresponding to 5′-AAUUCUCCGAACGUGUCACGU-3′ was used as a negative control. We introduced 50 nM of siRNA into cells with pRSV-β-gal expression plasmid and pGL3-CBA/JN or pGL3-DBA/2N reporter plasmid, using Superfect Transfection Reagent. Following 48 h of incubation, the cells were lysed and analyzed by Luciferase assay. The effects of siRNAs on the level of RBMX transcript were confirmed by Northern blot analysis as previously described [6]. We also carried out control experiments with a probe for 18S ribosomal RNA.

To confirm the specific effect of RNA interference for RBMX, siRNA from Dharmacon, Inc. (http://www.dharmacon.com/) was also used. We introduced 100 nM of siGENOME SMART pool reagent for Mouse RBMX into cells with pRSV-β-gal expression plasmid and pGL3-CBA/JN or pGL3-DBA/2N reporter plasmids using DharmaFECT 4 transfection reagent. Following 48 h of incubation, the cells were lysed and analyzed by Luciferase assay. The effects of siRNAs on the level of RBMX protein were also analyzed by Western blot analysis with anti-RBMX (hnRNPG) antibody as previously described [6]. We also carried out control experiments with anti-Nucleoporin p62 antibody (BD Biosciences).

3. Results

3.1. Partial purification of RBMX by DNA affinity chromatography and amino acid analyses

As previously reported [6], EMSA of nuclear protein extracted from the livers of the CBA/JN mice after an intake of the high-fructose diet revealed two specific bands recognizing oligonucleotides −453 to −480 bp of the upstream region of the SREBP-1c gene of the CBA/JN mice (Fig. 1a). These bands disappeared with the addition of an excess of unlabeled probe. These bands were detected only faintly when we used the oligonuleotides −453 to −480 bp of the upstream region of the SREBP-1c gene of the DBA/2N mice (Fig. 1b). To identify these proteins having the binding activity at the −453 to −480 bp region of SREBP-1c, DNA affinity chromatography was carried out as described in Materials and methods. The eluted fraction was applied to a gel for SDS/PAGE, and proteins were visualized by silver staining (Fig. 1c). Two bands

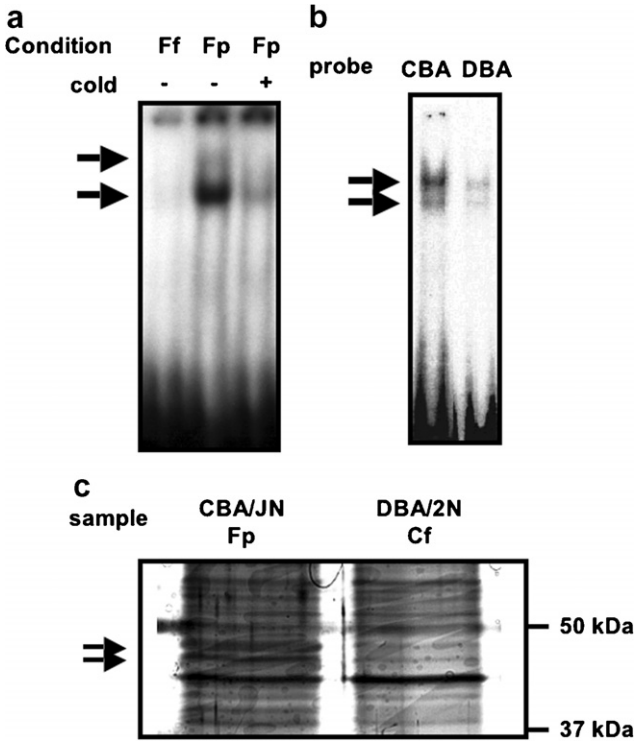

Fig. 1. (a) EMSA was performed using radiolabeled probe carrying the sequence at −453 to −480 bp of the 5′ flanking region of the SREBP-1c gene of the CBA/JN mice. The CBA/JN mice were fed high-fructose (*F*) diet in the overnight fasting state (f, fasting) or at 2 h after intake of diets (p, postprandial), and the nuclear proteins were extracted from the livers. Competition analysis was performed by adding the unlabeled probe with a 100-fold molar excess. The positions of two specific protein–DNA complexes (arrows) are indicated. (b) Effects of single nucleotide polymorphism on the mobility of the protein–DNA complexes. Radiolabeled probe carrying the sequence at −453 to −480 bp of the 5′ flanking region of the SREBP-1c gene of CBA/JN mice and DNA/2N mice were used. The nuclear proteins were extracted from the livers of CBA/JN mice who fed high-fructose diet 2 h ago. The positions of two specific protein–DNA complexes (arrows) are indicated. (c) SDS/PAGE analysis with proteins fractionated by chromatography on the DNA affinity resin. The arrows indicate the positions corresponding to two bands, of 42 and 43 kDa, which were strongly detected with the samples from CBA/JN mouse liver.

(42 and 43 kDa) were detected strongly with the sample from CBA/JN mice. On the other hand, these bands were detected only faintly with the sample from DBA/2N mice. In order to identify them, these bands were excised from the gel, digested with trypsin, and analyzed by MALDI-TOF MASS spectrometry. The resulting spectra were used to search for matching proteins in the NCBI database using the Mascot search program. Although the 43-kDa band is still being analyzed at present, the search for the CBA/JN specific 42-kDa band yielded a top score of 338 for RBMX (protein scores greater than 67 are significant; $P < 0.05$). The amino acid residues identified by MALDI were 10–22, 23–33, 31–41, 50–63, 50–67, 78–93, 94–101, 95–103, 102–110, 142–153, 149–161, 154–169, 162–177, 193–200, 201–214, 230–241, 256–265, 280–289, 290–306, 315–328, 329–338, 337–344, 345–352, 359–374 and 375–385. The nominal mass of the protein was 42 136 Da. Thus, it is suggested that RBMX is contained in the protein complex binding to the region between −453 and −480 bp of the SREBP-1c promoter.

3.2. Effects of anti-RBMX antibody and RNAi on EMSA

To confirm that RBMX is included in the bands seen in the EMSA experiment presented in Fig. 1, the effect of anti-hnRNPG (RBMX) antibody on EMSA was examined. As shown in Fig. 2a, the anti-RBMX antibody remarkably weakened the two bands, especially the upper band, while the anti-IgG antibody did not influence these bands at all. In addition, similar decreases in the signal of the two bands were observed when Fao cells were transfected with RBMX siRNA, as shown in Fig. 2b. Same extracts from control and RBMX siRNA treated cells bound equally well to Oct1 target sequence (Fig. 2c). Thus, it seems very likely that RBMX is included in these bands recognizing the region between −453 and −480 bp of the SREBP-1c promoter.

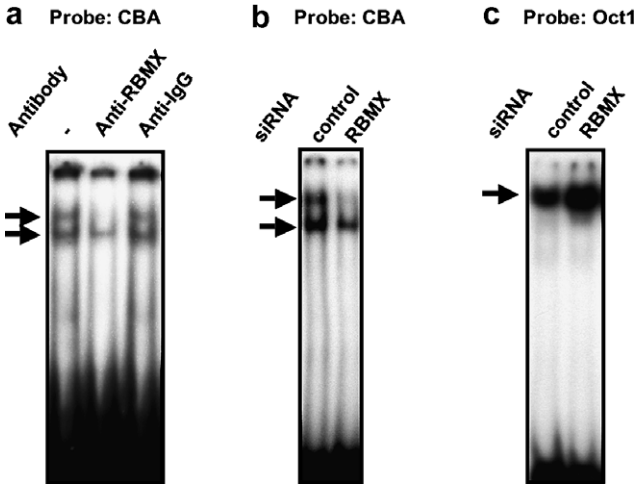

Fig. 2. (a) Effects of anti-RBMX antibody on the mobility of the protein-DNA complex. To 15 μl of the binding reaction mixture, 5 μl of 200-μg/ml anti-RBMX (hnRNPG) antibody was added. As a control, 5 μl of 200-μg/ml anti-IgG antibody was used. The arrows indicate the positions corresponding to the protein–DNA complexes. (b) Effect of RNAi with on the EMSA. Nuclear proteins were extracted from the transfected cells after 48 h. EMSAs were performed using probe carrying the sequence at −453 to −480 bp of the 5′ flanking region of the SREBP-1c gene of the CBA/JN mice. The positions of specific protein–DNA complexes (arrows) are indicated. (c) Control EMSAs with the same nuclear protein extracts were also performed using OCT-1 consensus oligonucleotides. The position of specific protein–DNA complexes (arrow) is indicated.

3.3. Effect of RBMX expression on SREBP-1c promoter activity

To investigate the influence of the amount of intracellular RBMX on SREBP-1c promoter activity, we constructed RBMX expression plasmids. We amplified by PCR the full-length cDNA of RBMX from total RNA of CBA/JN mouse liver and recloned it into the pcDNA3.1 expression vector. The effect of the RBMX expression plasmid on SREBP-1c promoter activity was analyzed using the luciferase reporter plasmid carrying the 616-bp SERBP-1c promoter region of the CBA/JN or DBA/2N mouse strain. The activity of SREBP-1c promoter from the CBA/JN strain was increased more remarkably than DBA/2N strain when the cells were cotransfected with the RBMX expression plasmid (Fig. 3a).

3.4. Effect of RNAi on SREBP-1c promoter activity

To examine the influence of the decreased expression of RBMX protein on SREBP-1c promoter activity, siRNAs (iGENE) were used. The activity of the SREBP-1c promoter was reduced to 30.8% in the cells cotransfected with the RBMX siRNA (Fig. 3b). Although the Fao cells are hepatic cells of rat origin, siRNA from Dharmacon, which was designed for mouse RBMX at another position of the sequence, showed the same effects on SREBP-1c promoter activity (data not shown). Fig. 3c and d depict results of Northern and Western blot analysis at 48 h after the lipofection of RBMX siRNA in Fao cells. The signals for the RBMX were decreased on the levels of both mRNA and protein. These effects of the RBMX siRNAs were sequence-specific, since control siRNA had no effect on the amount of RBMX, SREBP-1c promoter activity, or EMSA (Figs. 2b and c and 3b–d).

4. Discussion

In this report, we have identified RBMX as a protein recognizing the promoter region of SREBP-1c, where we have found a single nucleotide polymorphism relating to the response to high-fructose diet [6]. We confirmed RBMX as the protein binding to the region between −453 and −480 bp of SREBP-1c promoter by using two independent methods. Anti-RBMX antibody reduced the signals detected with the EMSA using the probe corresponding to the SREBP-1c promoter from −453 to −480 bp. In addition, these signals detected with EMSA were reduced by the specific suppression of RBMX with siRNA. Although these results clearly indicate that RBMX is involved in the fructose-induced signals detected with the EMSA using the probe corresponding to the SREBP-1c promoter from −453 to −480 bp, it is not clear at the present time whether RBMX can directly bind to this region. In fact, there is no report describing the presence of DNA binding domain in RBMX protein. It is possible that RBMX may regulate SREBP-1c promoter activity through another DNA-binding protein. The ability of RBMX to associate with other proteins has already been confirmed with hnRNPG and tra-2beta [7–9]. RBMX protein has a eukaryotic RNA recognition motif (RRM) at the N terminus [10]. Since some coactivator proteins such as PGC-1alpha and beta also have an

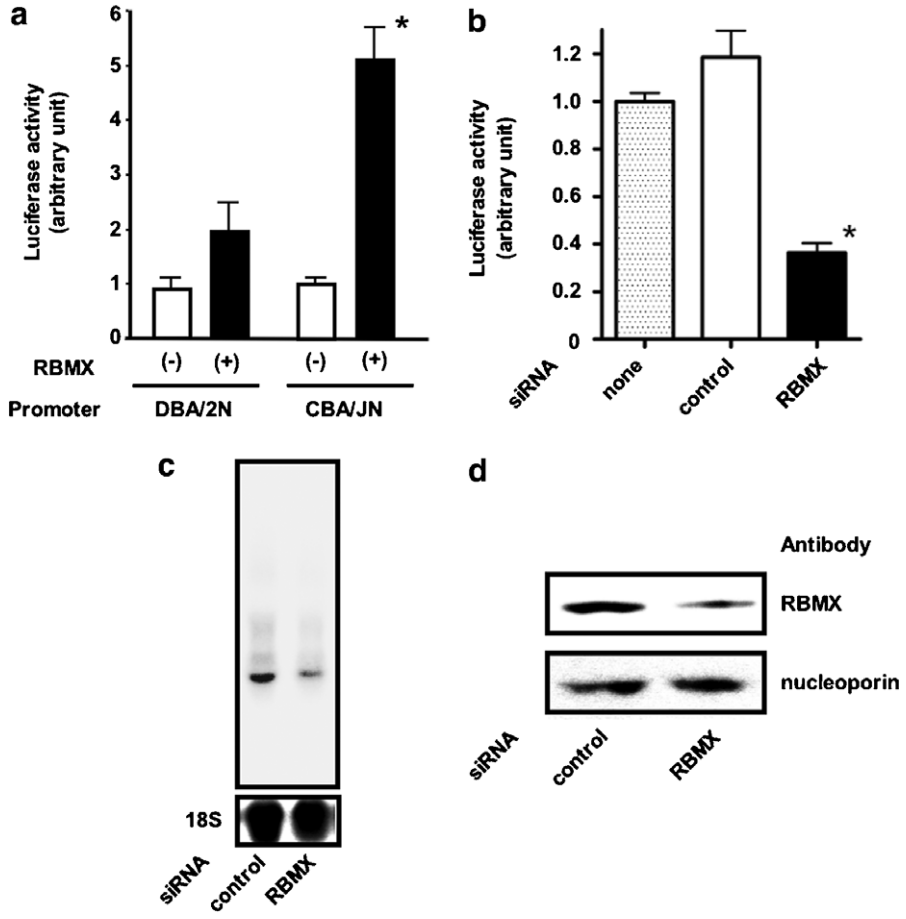

Fig. 3. (a) Effects of RBMX-expressing plasmid on the promoter activity of the 5′-flanking fragments of SREBP-1c gene prepared from the CBA/JN or DBA/2N mice. Fao cells were transfected with each reporter plasmid, reference plasmid, control vector or RBMX expression plasmid. Following 48 h of incubation, the cells were lysed and analyzed by luciferase assay. All luciferase activities were normalized against the activity of β-galactosidase. The data are expressed as the means ± S.E. ($n = 11$). *, $P < 0.01$ vs. the control. (b) Effects of siRNA targeting RBMX on the promoter activity of SREBP-1c gene. Fao cells were transfected with the reporter plasmid, pGL3-CBA/JN, reference plasmid and RBMX or control siRNA. Following 48 h of incubation, the cells were lysed and analyzed by luciferase assay. All luciferase activities were normalized against the activity of β-galactosidase. The data are expressed as the means ± S.E. ($n = 3$). *, $P < 0.01$ vs. the control. (c) Effect of RNAi on the level of RBMX transcript. Total RNA extract from cells which were lipofected with RBMX siRNA or control siRNA for 48 h was separated in 1.0% agarose/formaldehyde gel, blotted on to the nylon membrane, and hybridized with a radiolabeled RBMX probe or 18S rRNA probe as a control experiment. (d) Effect of siRNA on the level of RBMX protein. The nuclear proteins were extracted after 48 h of lipofection, and analyzed by Western blotting analysis with anti-RBMX (hnRNPG) antibody or anti-Nucleoporin p62 antibody as a control experiment.

RNA recognition motif [11–13], it may be possible that RBMX works as a coactivator.

As shown in the present study, the overexpression of RBMX by transfecting the expression vector increased the activity of SREBP-1c promoter while the reduction of the expression of RBMX with siRNA decreased the activity. These results strongly suggest that RBMX regulates the activity of the SREBP-1c promoter. Furthermore, it is possible that high-fructose diet induces the RBMX expression and controls the expression of SREBP-1c in the liver. Although we observed a significant increase in the RBMX signal detected with EMSA after high-fructose feeding, as shown in Fig. 1, no significant change in the mRNA expression in the liver was observed after high-fructose feeding (data not shown), suggesting the presence of functional activation of RBMX by high-fructose diet. The C-terminal domain of RBMX is composed of a high proportion of serine, arginine and glycine residues and is glycosylated [10]. There is also evidence that RBMX is modified in the cell by the addition of O-linked *N*-acetylglucosamine [14] and that this dynamic post-translational modification might regulate protein function in a manner resembling phosphorylation [15]. Thus, it is possible that RBMX may regulate SREBP-1c expression in the liver through its glycosylation in response to the high-fructose diet.

RBMX was first cloned as a cDNA encoding a nuclear 43-kDa glycoprotein identified as hnRNPG [16]. The mouse *hnrnpg* gene turned out to be a retroposon derived from *rbmx* gene because of its lack of intronic sequence [17]. RBMX is also known as the X homologue of the candidate spermatogenesis gene RBMY (for RNA-binding motif gene, Y chromosome) [17,18]. RBMY is expressed exclusively in the testis [19,20], whereas RBMX is ubiquitously expressed in adult mice [17] and in humans [14,21], though it may be differentially expressed in a tissue-specific manner [22]. The widespread expression pattern of RBMX suggests that it may exert its influence on general processes; however, there is little information on the role of RBMX in mammals. Recently, the function of RBMX during embryonic development, in particular of the brain, in

zebrafish was reported [23]. Although zebrafish *rbmx* is maternally expressed and widely distributed in the early stages of embryogenesis, the transcripts were found to be localized in the brain, branchial arches and liver primordium in the late stages. This suggests that RBMX has an important function specifically in the liver in addition to the brain.

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